SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Recombinant plasmids-BACE1, PS1 M146V and D385A plasmids was obtained from Dr. Luciano D'Adamio, Albert Einstein College of Medicine, Bronx, New York, USA. PS1 wild type and PS1 mutant L392V (1) were obtained from skin fibroblasts of carrier patients, PS1 mutant S170F (2) was obtained from brain tissue of a carrier patient. Total RNA was extracted using the Trizol method (Invitrogen, Carlsbad, USA) and cDNA was synthesized starting from 3μg of RNA using random primers (SuperScript II kit, Invitrogen, Carlsbad, USA). PS1 was amplified by PCR (Primers: FW 5'-GCT CCA ATG ACA GAG TTA CCT GC-3'; REV 5'-GAA ACA TCC ATG GGA TTC TAA CCG-3'). The PCR products were purified from the agarose gel and cloned using a PCR cloning kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. PS1 mutants and wild type clones were identified and confirmed by direct sequencing; the PCR products were sub-cloned into the pcDNA3.1 vector. PS1 mutant M146V (3) was obtained by site direct mutagenesis and likewise cloned into the pcDNA3.1 vector. *AICD 50, 51, 57 and 59 constructs* were cloned from full length APP plasmids using the following primers: BamHI-AID50S2 5'- CGg gat ccA CCa tgG TGA TGC TGA AGA AG-3'; BamHI-AID51S2 5'- CGg gat ccA CCa tgT TGG TGA TGC TGA AGA AG-3'; BamHI-AID57S2 5'- CGg gat ccA CCa tgA CAG TGA TCG TCA TCA C-3'; BamHI-AID59s2 5'- CGg gat ccG CCA CCa tgA TAG CGA CAG TGA TCG TC-3'; AID-Stop-XhoI AS1 5'- CCG ctc gag CTA GTT CTG CAT CTG CTC AAA G-3'. Fragments were then cloned into the MCS of pcDNA3.1.

Aβ ELISA and βAPPs levels in HEK-293 APPwt, SH-SY5Y and M17 PS1 Stable cells-The amounts of secreted Aβx-40 and Aβx-42 were evaluated by sandwich ELISA (IBL, Gumna, Japan). Media of HEK-293 APPwt cells, harvested after an overnight, were diluted 1:40 in EIA buffer and processed using a kit specific for both Aβ species, according to manufacturer's protocol. Media of PS1 stable neuroblastoma cells were collected also after an overnight conditioning, but not diluted. The kits are solid phase sandwich ELISA using plates pre-coated with the specific polyclonal anti-human Aβ antibody (raised against residues 38-42 or 35-40). An HRP-conjugated monoclonal anti-human Aβ antibody (residues 11-28) is also supplied. Both assays show a linear reactivity within the range of concentration 7-1000 pg/ml for both Aβ species. Aβ concentration was determined using a Benchmark Microplate Reader and evaluated by Microplate Manager Version 5.1 Software (Biorad, Hercules, CA, USA). For quantification of βAPPs, 50μg-corresponding proteins of culture medium were resolved on 7,5% SDS-PAGE gels, transferred to PVDF membranes (Amersham Biosciences, Buckingam Shire, UK), and probed with an antibody specific for βAPPs (IBL, Gumna, Japan).

PS1 M146V knock-in mice generation-To obtain mutant PS1 knock-in mice, a targeting vector containing a neomycin resistance cassette and herpes simplex virus thymidine kinase gene was utilized in constructing this mutant. A mutagenized DNA sequence of exon 5 of the mouse PS1 gene was targeted for the PS1 allele. The construct was electroporated into 129X1/SvJ x 129S1/Sv-derived R1 embryonic stem (ES) cells. Correctly targeted ES cells were injected into recipient blastocysts. The resulting chimeric male animals were bred to C57BL/6 females to produce mice heterozygous for the mutation. These mice were then bred to a CMV-Cre transgenic strain to delete the neomycin cassette from the targeted allele (4).

PS1 M146V knock-in mouse brain tissue-Brains (n=8, 4 males, 4 females) of knock-in PS1 mutant mice (2-5 months old), bearing the PS1 M146V mutation, and of age-matched littermate control mice (n=7, 4 males, 3 females) were used.

Human brain tissue-We used samples of frontal cortex from 11 FAD cases bearing 10 different PS1 mutations and expressing various phenotypes (Suppl. Table 1), and of 10 SAD cases. Samples of frontal cortex from 12 neurologically normal age-matched subjects were used as controls (provided by Dr. Pierluigi Gambetti, Case Western Reserve University, Cleveland, OH, USA).

Evaluation of BACE1 activity-Proteins were extracted, from cells or brain tissue, with the provided extraction buffer according to the manufacturer's protocol (kit form R&D Systems, Inc., Minneapolis, MN, USA). The method is based on the cleavage of a BACE1 specific peptide (REEVNLDAEFKR), conjugated with the fluorescent reporter molecules EDANS and DABCYL. Cleavage of the peptide by the BACE1 separates EDANS and DABCYL from each other, with the release of a fluorescent signal; the level of BACE1 enzymatic activity is proportional to the intensity of signal The peptide provided in the kit is specific for BACE1, since its sequence bears

the two residues, upstream to the Aβ sequence, corresponding to the APP Swedish mutation. This sequence makes the peptide not sensitive to other proteases, such as the cathepsin family. Moreover, the pH of the extraction buffer inhibits the activity of cathepsins (5). To test for specificity, BACE1 Inhibitor IV (Calbiochem, Darmstadt, Germany) was administered 15nM to HEK293 cells at the time of plating, for 16 hours, and after transfection for another 12 hours. BACE1 activity was assayed 12 hours after transfection.

Aβ peptides aggregation, TEM and western blot-Aβ peptides were resuspended as described in the Experiemntal Procedure section, from -80°C frozen aliquots. Fresh preparation, to be kept in culture for 1 or 6 hours, were resuspended at 1μM in culture medium, vortexed for 30", recollected by brief centrifugation and left in culture hood for the appropriate time. To obtain oligomers, dried peptides were resuspended to 100μM in double distilled water and kept at room temperature (c.a. 23°C) for 72 hours. To obtain fibrils, dried peptides were resuspended to 100μM 10mM HCl and kept at 37°C for 72 hours. For *TEM analysis*, 5 microliters of sample were put on carbon-coated formvar copper grid for 10 minutes, dried, and then stained with 2% PhosphoTungstic acid. Samples were then analized by trasmission electron microscopy CM10 (Fei Company, Hillsboro, OR, USA)." For *western blot*, an aliquot of the peptides was run, in 1x tris-tricine sample buffer + 15% β-Mercaptoethanol, onto a 10-18% tris tricine maxi gel, transferred upon a 0,2 μm nitrocellulose membrane (Biorad, Hercules, CA, USA). Membrane was boiled in microwave, soaked in PBS without Ca^{++} and Mg^{++}, for 3x5' at 450W, blocked in 5% milk in TBS, and probed with the anti APP antibody 6E10 (Signet Lab, Dedham, MA, USA) 1/500 in 20% Superblock (Pierce, Rockford, IL, USA) in TBS. Blot was developed using Pierce's West pico with 30% West Dura addition.

Antibody in-culture treatment of cells-Antibody treatment of HEK 293 APPwt cells was carried out 16-18 hours after plating, and 1 hour after cell transfection with either pcDNA3.1, PS1 WT, or PS1 S170F. Different antibodies were added to the culture medium: 1A10 monoclonal specific for Aβ40 (1:320, IBL, Gumna, Japan), 21F12 monoclonal specific for Aβ42 (1:480, Elan Inc., San Francisco, CA, USA) and a monoclonal against β-Actin (1:2000, Sigma, St. Louis, MO, USA). Cells were harvested after 3 hours treatment, for RNA extraction.

Primary neuronal cultures-Six well culture plates were coated with 15μg/mL Poly-L-Ornithine (Low Molecular Weight, Sigma, S.Louis, MO, USA) for 45 minutes at room temperature. Poly-L-Ornithine was the aspirated and wells were soaked with 4μg/mL mouse Laminin (Invitrogen, Carlsbad, Ca, USA), for 12-16 hours in a cell culture incubator at 37°C, 95% humidity and 5% CO2. Eight weeks old FVB female mice were bred with age matched male mice for 3 days. Pregnancy was ascertained according to vaginal plug and weight gain of the females. Females were sacrificed by cervical dislocation, after sedation with isoflurane, at 17.5 days of gestation. Foetuses were processed separately, in order to obtain pure transgenic cultures. Genotyping was carried out as described, by isolating tail DNA. Forebrains were dissected in ice cold HBSS (Invitrogen, Carlsbad, Ca, USA) + 0.5% w/v D-Glucose (Sigma, S.Louis, MO, USA) and 25mM Hepes (Invitrogen, Carlsbad, Ca, USA), under a dissection microscope (Zoom 2000, Leica, Wetzlar, Germany). Dissociation was carried out in ice cold dissection medium plus 0.01%w/v Papain (Worthington, Lakewood, NJ, USA), 0.1%w/v Dispase (Roche, Indianapolis, IN, USA) and 0.01% w/v DNase (Worthington, Lakewood, NJ, USA), first by means of sterile razor blades, then by serial pipetting with flamed sterile glass Pasteur pipettes, and incubation at 37°C twice for 15 minutes. Cells were then spun down at 220g for 5' at 4°C, resuspended in Neurobasal Medium with 2% B27, 1mM Na Pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, 2mM Glutamax (all from Invitrogen, Carlsbad, Ca, USA), filtered through a 40μm cell strainer (Fisher, Waltham, MA, USA), counted and plated on coated 6well plates at a density of about 750.000 cells/well. Culture medium was completely replaced after 16-20 hours, and new medium (30% of starting volume) was added every 3 days until needed. For *immunostaining*, cells were washed in TBS once and fixed with 4% PFA for 30' at room temperature, washed again and permeabilized with 0.2% Triton X-100/TBS for 10' on ice, and cold methanol for 5' on ice. Blocking of aspecific antigenic sites was performed with 10% Goat Serum/0.2% Triton X-100/TBS for 1hr at RT. Primary antibody against MAP2 (Sigma, S.Louis, MO, USA, monoclonal clone HM-2, 1/500), and secondary antibody anti-mouse-HRP (Southern Biotech, Birmingham, Alabama, USA) were diluted in in 5% Goat Serum/0.1% Triton X-100/TBS for 90' at room temperature. All washes

in between and after antibodies incubations were 2x10' with TBS pH7.6/0.2% Triton X-100. The reaction was visualized by DAB Chromogen (DAKO, Glostrup, Denmark). Sections were then mounted on a glass slide, dehydrated and covered with DPX (Sigma, S.Louis, MO, USA).

Quantitative Real Time PCR analysis-Total RNA was extracted from T25 flasks or 6-wells of cultured cells and neurons, from frozen brain tissues or from newly sacrificed transgenic pups, using the TRIZOL method, according to the manufacturer's protocol (Invitrogen, Carlsbad, Ca, USA). Three μg of total RNA were reverse-transcribed using random primers. Primers and probes for BACE1 were designed with the aid of Primer Express software (Applied Biosystems, Foster City, CA, U.S.A.). To avoid amplification of contaminating genomic DNA, primers and probes were located on adjacent exons. Set primers and probe for BACE1: forward (human and mouse) 5'- TGG AGG GCT TCT ACG TTG TCT T-3', reverse (human) 5'-GCT GCC GTC CTG AAC TCA TC-3', reverse (mouse) 5'-CAT CAT GGA AGG TTT CTA TGT CGT CTT C-3', probe (human and mouse) 6fam-5'- TTG GCT TTG CTG TCA GCG CTT GC- 3' Tamra. Primers and probes for beta-actin (human and mouse) were obtained from a pre-developed assay-on-demand (Applied Biosystems, Foster City, CA, U.S.A.). Five μl of the resulting cDNA dilution were used for quantitative PCR amplification performed, in duplicate, on the Prism 7900HT Instrument (Applied Biosystems, Foster City, CA, U.S.A) with the fluorescent TaqMan method. The BACE1 mRNA quantities were normalized to the control gene and were expressed in relation to a calibrator sample. The levels of transcripts BACE1 and beta-actin in each sample were determined using the standard curve. The standard curve was obtained with serial dilutions (10^6-10 molecules) of the calibrator, control plasmids containing cloned sequences of ABL gene (Ipsogen, Marseille, France).

MTT assay, ROS and GSSG/GSH determination-For MTT, ROS and GSSG/GSH assays, SH-SY5Y cells were plated and cultured as described in the Experimental Procedures section of the manuscript and treated with Aβ species or H_2O_2 20μM, in a 96 well plate format for MTT and on 24 well format coverslips for the other tests. Cells were then washed and incubated with MTT solution for 3 hours in incubator (1/10 in culture medium; stock: 5mg/mL in PBS; Sigma Chemical Company, St. Louis, USA). Solubilizing solution (10% SDS in 0.01N HCl) was then added (same volume as MTT-medium) and incubated over night at 37°C, on a shaker. The next day wells were read at 570nm with background subtraction at 620nm, using a Benchmark Microplate Reader, with Microplate Manager Version 5.1 Software (Biorad, Hercules, CA, USA). Intracellular generation of ROS was detected by detecting the conversion of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, 5 μM), internalized by cells and de-acetylated by esterase, into the corresponding fluorescent derivative. Cells were observed and photographed under a Zeiss fluorescence microscope (6). Antioxidant levels in the cytosolic fractions were evaluated in terms of GSSG/GSH ratio, by the Owens and Belcher (1965) method. A mixture was directly prepared in a cuvette: 0.05 mmol/L Na phosphate buffer, pH 7.0; 1 mmol/L EDTA, pH 7.0; and 10 mmol/L 5-5'-Dithiobis-(2-nitrobenzoic acid) plus an aliquot of the sample. GSH content was evaluated after 2 min at 412 nm and expressed as μg/mg protein. Suitable volumes of diluted GSH reductase and of NADPH were then added to evaluate the total GSH level. The ratio between GSSG and GSH content is considered a measure of antioxidant status.

AICD transgenic mice-Transgenic AICD mice were generated as described recently (7). mRNA was obtained form one hemisphere of 4 weeks pups of 7 transgenic mice and their corresponding littermates, as described, with the Trizol method, and retro-transcribed to cDNA as described. Real time PCR for BACE1 was performed as described.

AICD semiquantitative PCR-cDNA form cells transfected with AICDs or pcDNA3.1, obtained as described, was used as a template for PCR with the following primers: S1 5'-ATGCTGAAGAAGAAACAGTAC -3'; AS2 5'-GGATTTTCGTAGCCGTTCTGCTGC –3'.

Chemicals-All chemicals are from Sigma, S.Louis, MO, USA, unless otherwise stated.

Statistical analysis-Data were statistically analyzed using the unpaired Student's t-test or ANOVA, followed by the Bonferroni post-test and by the study of linear correlation with its correlation coefficient where applicable.

REFERENCES

1. Campion, D., Flaman, J. M., Brice, A., Hannequin, D., Dubois, B., Martin, C., Moreau, V., Charbonnier, F., Didierjean, O., Tardieu, S., and et al. (1995) *Hum Mol Genet* **4**, 2373-2377
2. Snider, B. J., Norton, J., Coats, M. A., Chakraverty, S., Hou, C. E., Jervis, R., Lendon, C. L., Goate, A. M., McKeel, D. W., Jr., and Morris, J. C. (2005) *Arch Neurol* **62**, 1821-1830
3. Clark, R. F., Cruts, M., Korenblat, K. M., He, C., Talbot, C., Van Broeckhoven, C., and Goate, A. M. (1995) *Hum Mol Genet* **4**, 1347-1354
4. Guo, Q., Fu, W., Sopher, B. L., Miller, M. W., Ware, C. B., Martin, G. M., and Mattson, M. P. (1999) *Nat Med* **5**, 101-106
5. Gruninger-Leitch, F., Schlatter, D., Kung, E., Nelbock, P., and Dobeli, H. (2002) *J Biol Chem* **277**, 4687-4693
6. Ravindranath, V. (1994) *Methods Enzymol* **233**, 610-619
7. Giliberto, L., Zhou, D., Weldon, R., Tamagno, E., De Luca, P., Tabaton, M., and D'Adamio, L. (2008) *Mol Neurodegener* **3**, 12